TRC Grants #6 & 162

Reprinted from PLANT PHYSIOLOGY, Vol. 34, No. 2, March, 1959, pages 123-127.
(PRINTED IN U.S.A.)

DARK FIXATION OF CO_2 BY TOBACCO LEAVES[1, 2, 3]

GEORGE KUNITAKE, CLYDE STITT AND PAUL SALTMAN

DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, SCHOOL OF MEDICINE, UNIVERSITY OF SOUTHERN CALIFORNIA, LOS ANGELES 7, CALIFORNIA

The ability of non-succulent plants to fix CO_2 in the absence of light has been previously demonstrated by several investigators (2, 5, 11, 17, 21). However, the succulents have received the greatest attention because of the large amounts of CO_2 incorporated into organic acids in the dark.

Although these earlier reports strongly suggested that dark fixation of CO_2 is an ubiquitous phenomenon in plants other than succulents, the nature of the biochemical reactions involved in this process has not been critically examined. Concurrently with the studies carried out in our laboratory on succulent metabolism (12, 13) we have investigated non-succulent dark CO_2 metabolism in *Nicotiana tabacum*. Studies concerning the nature of the initial carboxylation reaction and the fate of the fixed $C^{14}O_2$ during the subsequent metabolic reactions in the absence of light were carried out with excised tobacco leaves. These studies show that the pathways for the dark metabolism of CO_2 by succulent and non-succulent leaves appear to be fundamentally the same.

MATERIALS AND METHODS

The methods used in the study of the dark fixation of CO_2 in excised tobacco leaves are the same as those described in detail in earlier communications (12). *Nicotiana tabacum* (var. Hicks) plants used in these experiments were grown at the Earhart Plant Research Laboratory, Division of Biology, California Institute of Technology, Pasadena, California and most generously supplied by Dr. H. R. Highkin. Immediately before use, approximately 1 g of young leaves (5 to 7 cm long) were taken from the apex of the plants. The leaves were placed in an apparatus which permits exposure to $C^{14}O_2$ in total darkness. After equilibration of the leaves for 5 minutes in the dark to remove any transient reducing compounds of photosynthesis, $C^{14}O_2$ generated from 5.0 mg $BaC^{14}O_3$ (specific activity 120 μc/mg) was admitted into the chamber. After suitable exposure to $C^{14}O_2$, the reaction was terminated by homogenization in boiling 80 % ethanol.

The ethanol homogenate was filtered, the filtrate extracted with Skellysolv A, and the extract concentrated to a volume of 3 ml under reduced pressure. Concentrated alcoholic extracts were separated by two dimensional paper chromatography, phenol (80) : water (20), (w/w) in the 1st direction and *n*-butanol (74) : acetic acid (19) : water (50), (v/v/v) in the 2nd direction. Compounds were located with the appropriate sprays: amino acids with ninhydrin in collidine spray of Levy and Chung (9), organic acids with a mixed indicator spray of 3 g brom-phenol blue and 1 g methyl red per liter of 95 % ethanol. Radioactive compounds were located by radioautograms made by exposing the chromatograms to "no-screen" x-ray film. Activity of each compound was measured directly on the paper using an end-window Geiger tube.

2,4-Dinitrophenylhydrazones were prepared according to a modified procedure described by Ranson[4], in order to trap the α-keto acids which would otherwise be lost during the preparation and the subsequent chromatographic analysis of the plant extract. Approximately 8 g of leaves, with the midribs removed, were exposed to $C^{14}O_2$ in the absence of light for 10 minutes. The reaction was stopped by homogenization of the leaves with 20 ml of ice cold 5 *N* H_2SO_4. The homogenate was immediately filtered and the residue washed with 5 ml of 5 *N* H_2SO_4. Twenty-five ml of a saturated solution of 2,4-dinitrophenylhydrazine in 5 *N* H_2SO_4 was then added to the filtrate and placed in the cold for 24 hours to permit the formation of the 2,4-dinitrophenylhydrazones. The hydrazones were extracted from the acid solution with three 30-ml portions of ethyl acetate. The ethyl acetate was then extracted with three 30-ml portions of ice cold 10 % Na_2CO_3. The combined 10 % Na_2CO_3 extracts were adjusted to pH 1.0 with ice cold 5 *N* H_2SO_4 and extracted with three 20-ml portions of ethyl acetate. The combined ethyl acetate extracts were dried over anhydrous Na_2SO_4 and concentrated to a volume of 2 ml under reduced pressure. The 2,4-dinitrophenylhydrazone derivatives were chromatographed on buffered paper by the method of Isherwood and Cruikshank (7) and identified by co-chromatography with authentic samples. Radioautograms were prepared as above. The activity of each compound was measured directly on the paper using an end-window Geiger tube.

The enzyme, phosphoenolpyruvate carboxylase, was prepared from acetone powder of tobacco leaves and assayed by the method described by Bandurski and Greiner (1). The acetone powder was prepared by homogenizing 50 g of tobacco leaves with 500 ml of acetone in a Waring blendor at —20° C. The homogenate was immediately filtered through a Buchner

[1] Received August 7, 1958.

[2] This investigation was supported by a research grant from the Tobacco Industry Research Committee. The facilities of the Allan Hancock Foundation were generously provided.

[3] A preliminary report was presented at the meeting of the American Society of Plant Physiologist, August 26–30, 1956, University of Connecticut, Storrs, Connecticut.

[4] Personal communication.

funnel and washed with 50 ml of —20° C acetone. The powdered residue was dried over PCl_5 under vacuum. The dried acetone powder was stored in a tightly sealed bottle at 0° C. The enzyme was prepared by extracting 0.2 g of the acetone powder with 5.0 ml of 0.005 M pH 7.5 TRIS buffer, in the cold. After centrifugation this extract was used directly.

Results and Discussion

Exposure of the tobacco leaves to $C^{14}O_2$ in the dark for periods from 6 seconds to 5 hours, resulted in the sequential incorporation of the radioactive carbon into several compounds, principally organic and amino acids. Table I shows the total amount of radioactivity

Table I

Rate of the Dark Fixation of $C^{14}O_2$ By Leaves of Nicotiana, Tabacum and Bryophyllum Calycinum

Time dark exposure to $C^{14}O_2$ (min)	cpm/mg of leaves/mg $BaCO_3$*	
	N. tabacum	B. calycinum
1	9	6
5	22	10
15	50	23
30	79	109
60	124	407
120	209	2,280

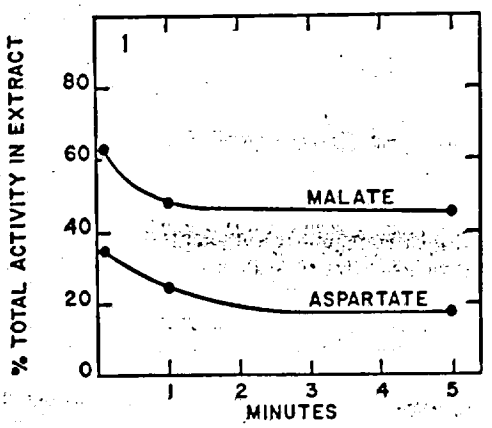
Leaves of tobacco and Bryophyllum were exposed independently to $C^{14}O_2$ in the dark for the indicated time intervals. The reaction was terminated by homogenizing with boiling 80 % ethanol, and an aliquot of the homogenate assayed for radioactivity.

* Samples were counted within a statistical error of 5 %.

incorporated by tobacco and Bryophyllum leaves exposed to $C^{14}O_2$ in the dark for various intervals. It appears that tobacco can fix CO_2 more rapidly in the initial stages of the dark carboxylation reaction. However, after the 1st 15 minutes the rate in the Bryophyllum seems to surpass that found in the tobacco leaves.

Malate and aspartate were the major detectable radioactive products on chromatograms of tobacco leaves exposed to $C^{14}O_2$ for 6 seconds. However, traces of citrate were also observed. The amount of citrate accounted for less than 0.01 % of the total activity present. The percent of total activity found in malate and aspartate after successively longer periods of exposure to $C^{14}O_2$ are plotted in figure 1. The extrapolation of these values to zero time strongly suggests the presence of a common precursor to malate and aspartate, probably oxaloacetate. To test this hypothesis, oxaloacetate was isolated and identified by cochromatography as the 2,4-dinitrophenylhydrazone derivative from tobacco leaves exposed to $C^{14}O_2$ in the dark for 10 minutes. It contained considerable radioactivity.

Since these results are similar to those found for succulent leaves (12, 13), the enzyme mediating the initial fixation of CO_2, phosphoenolpyruvate carboxylase, was extracted, and the data from a typical enzymatic assay are presented in table II. From these results it would appear that the initial carboxylation reaction is identical in both *Nicotiana tabacum* and *Bryophyllum calycinum*. It is suggested that figure 2 represents the initial reactions involved in the dark



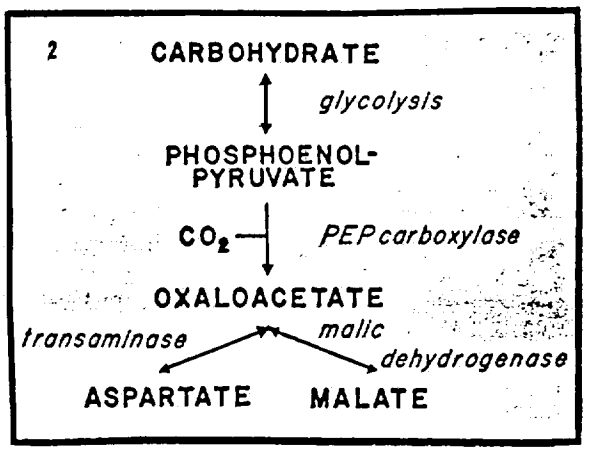


Fig. 1. Radioactivity of malate and aspartate expressed as percent total activity in extract as a function of time.

Fig. 2. Initial reactions involved in the dark fixation of CO_2 by tobacco leaves.

Table II

Phosphoenolpyruvate Carboxylase Activity in TRIS Buffer Extracts of Acetone Powder Prepared from Tobacco Leaves

Condition	cpm fixed in reaction mixture
Enzyme + phosphoenolpyruvate	10,600 ± 530
Enzyme — phosphoenolpyruvate	400 ± 20
Boiled + phosphoenolpyruvate enzyme	0

Each tube contained 60 micromoles TRIS hydrochloride pH 7.5, 20 micromoles $MgSO_4$, 100,000 cpm $NaHC^{14}O_3$, 0.2 ml enzyme, 6 micromoles phosphoenolpyruvate, total volume 1.5 ml. Incubated 60 minutes at 37° C. Reaction stopped with 0.1 ml 1 *N* HCl and the unreacted $C^{14}O_2$ removed by bubbling N_2 through the mixture. An 0.2-ml aliquot of the reaction mixture was counted with an end-window Geiger tube.

fixation of CO_2 in tobacco leaves. The isolation of labeled malate from young tobacco leaves exposed to $C^{14}O_2$ in the dark by Stutz and Burris (17) gives further evidence for the presence of a carboxylation reaction. Mazelis and Vennesland (10) have shown that the enzymes phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase are widely distributed in plant tissues. These results suggest the general occurrence of a mechanism for the dark fixation of CO_2 in leaves of higher plants similar to those we have proposed for both *Bryophyllum calycinum* (12) and *Nicotiana tabacum*. Vickery and Pucher (21) have proposed the presence of a similar reaction to account for the accumulation of citrate in tobacco leaves cultered in bicarbonate solutions in the dark. Incorporation of bicarbonate into citrate could take place via an initial carboxylation reaction to form a 4-carbon dicarboxylic acid which subsequently condenses with acetate.

The suggestion has been made by Bradbeer et al (3) that the initial carboxylation in the dark CO_2 fixation is on ribulose diphosphate. This unstable β-keto intermediate is immediately cleaved to phosphoglycerate and is subsequently metabolized to the 3-carbon acceptor phosphoenolpyruvate. This compound is then carboxylated to form the 4-carbon dicarboxylic acid. We have not been able to identify labeled phosphoglycerate in our short term experiments in either tobacco or Bryophyllum leaves exposed to $C^{14}O_2$ in the absence of light. However, after 5 minutes of dark fixation radioactivity can be detected in phosphoglycerate. It is possible that the age or the previous condition of the leaves could account for the differences in our observations.

Excised leaves of *Nicotiana tabacum* were exposed to $C^{14}O_2$ in the dark for periods as long as 5 hours. A typical radioautograph is presented in figure 3. The rates at which the compounds incorporate the radiocarbon from $C^{14}O_2$ are listed in table III. The radio-

TABLE III

PRODUCTS FROM THE DARK FIXATION OF $C^{14}O_2$ BY N. TABACUM LEAVES

COMPOUNDS	1 MINUTE	5 MINUTES	15 MINUTES	30 MINUTES
Malate	47.1	46.4	55.2	59.8
Citrate	12.1	11.6	3.6	5.3
Isocitrate	2.3	1.5	1.8	2.6
Succinate	1.9	4.7	5.8	4.5
Fumarate	0.3	1.0	1.1	1.1
Aspartate	23.9	17.4	13.2	8.0
Alanine	2.3	4.3	10.0	13.5
Glutamate	3.2	1.2	3.4	1.5
Serine	2.4	0.8	0.8	0.4
Glycine	0.6	0.2	0.4	0.2
Glutamine		Trace	1.1	1.3
Histidine			Trace	Trace
Proline			Trace	Trace
Threonine			Trace	Trace
Hydroxy-proline			Trace	Trace
Arginine			Trace	Trace

Concentrated alcoholic extracts of tobacco leaves exposed to $C^{14}O_2$ for the indicated intervals were separated by 2-dimensional paper chromatography as described. Following location and identification of radioactive compounds by radioautography the activity in each compound was measured with an end-window Geiger tube.

Activities are expressed as percent of total activity counted on the chromatogram. Samples were counted within a statistical error of 5%. Those compounds indicated as "Trace" had less than 0.1 % of the total activity.

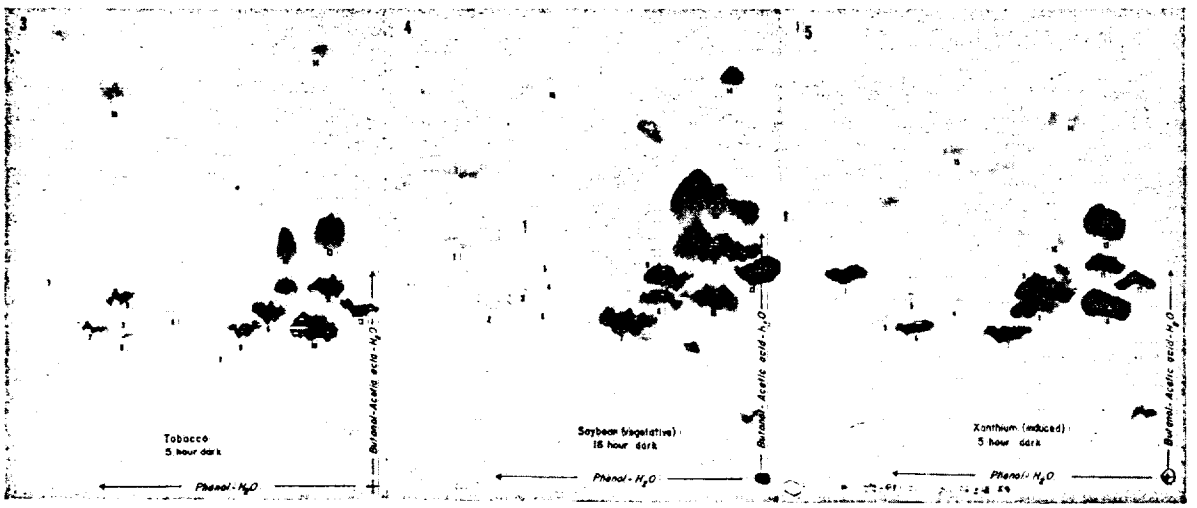


FIG. 3 Radioautogram of ethanol extract from tobacco leaf after 5 hours of dark $C^{14}O_2$ fixation. Compounds which have been identified are: 1. proline, 2. arginine, 3. hydroxy-proline, 4. threonine, 6. glutamine, 7. asparagine, 8. serine-glycine, 9. glutamate, 10. aspartate, 11. unidentified ester of malic acid, 12. *iso*citrate-citrate, 13. malate, 14. fumarate, 15. succinate, 16. glycerate.

FIG. 4. Radioautogram of extracts from soybean showing the absence of carbohydrates and phosphoralated derivatives. Compounds identified are numbered as in figure 3.

FIG. 5. Radioautogram of extracts from xanthium showing the absence of carbohydrates and phosphoralated derivatives. Compounds identified are numbered as in figure 3.

activities are expressed as percent of total activity counted on the paper at given time intervals.

In addition to the listed compounds, the labile α-keto acids, pyruvate, oxaloacetate and α-ketoglutarate were also isolated and identified as their 2,4-dinitrophenylhydrazones. There was no detectable activity present in glyoxylate. We have been unable to detect any radioactivity associated with the carbohydrate or phosphorylated carbohydrate fractions in tobacco leaves exposed to $C^{14}O_2$ in the dark for 5 hours. Sen and Leopold (14) report a rather significant carbohydrate fraction, 7 to 77 %, of the $C^{14}O_2$ fixed in the dark after 16 hours by Biloxi soybean, cockelbur, and Wintex barley. Our attempts to confirm the results of Sen and Leopold by exposing detached leaves of the above plants to $C^{14}O_2$ for 16 hours have thus far been unsuccessful. Radioautographs of the extracts from soybean, cockelbur are shown in figure 4. It would appear in leaves and other organs of higher plants that light is necessary for the generation of the reducing power needed to reverse the glycolytic pathway. However, germinating castor beans (16) can incorporate $C^{14}O_2$ into carbohydrates in the absence of light under conditions where fat is transformed into sugar.

The presence of an active Krebs cycle in photosynthesizing tissues has been an elusive problem for a long time. Recently, Smillie (15) has resolved this problem by preparing active cytoplasmic particles from green pea leaves, thus clearly establishing for the first time the operation of an active Krebs cycle in green leaves. We have been able to identify and isolate essentially all of the intermediates of the Krebs cycle with the exception of cis-aconitate. Although we have not demonstrated directly the enzymatic systems present in the cycle, the incorporation of $C^{14}O_2$ into cycle acids and related amino acids suggests that the cycle is operative in tobacco leaves.

The results obtained by Vickery et al (20) in their studies on the metabolism of excised tobacco leaves in various culture media strongly support the operation of an active Krebs cycle. Malate, succinate, and fumarate were shown to be equally effective as precursors in the formation of citrate. With succinate as a substrate significant amounts of malate accumulated. A decrease in malate concentration was observed when fumarate was used. Vickery et al (20) feel that these observations are not compatible with the operation of the Krebs cycle since fumarate should be a mandatory intermediate in the conversion of succinate to malate. It is possible to formulate another hypothesis consistent with the operation of the Krebs cycle to explain the above results. If one considers that malate accumulation is controlled by the ability of the plant cell to transport this acid into a metabolically inactive area of the cell such as the vacuole, it is possible that the addition of fumarate in high concentrations could inhibit this transport mechanism resulting in a decreased concentration of malate. Preliminary experiments in our laboratory indicate that differential vacuolar transport systems are operative. This inhibition of malate accumulation by fumarate would not in any way affect the metabolic pathway leading to the formation of citrate. Vickery et al (20) report that samples cultures in succinate did not accumulate any detectable fumarate. Therefore, succinate is converted to citrate and the accumulation of malate occurs in the absence of the inhibitory effects of abnormally high concentration of fumarate.

Glycolate did not incorporate radiocarbon in the dark when leaves were exposed to $C^{14}O_2$ for as long as 5 hours in the absence of light. It was reported earlier by Benson and Calvin (2) that in Chlorella, Scenedesmus and barley leaves labeled glycolate was found only during photosynthesis. Clagett, Tolbert and Burris (4) demonstrated that glyoxylate is formed by the direct oxidation of glycolate in plants. Kenton and Mann (8) found that glyoxylate can be oxidized to oxalate in tobacco leaves. Our inability to find labeled glycolate, glyoxylate or oxalate in excised leaves of tobacco exposed to $C^{14}O_2$ in the dark would appear to be in complete agreement with the known biosynthetic pathways of these compounds in leaves of higher plants. Although oxalate is one of the major acid components of tobacco leaves (19), we have been unable to demonstrate the incorporation of radiocarbon from $C^{14}O_2$ into this acid after 5 hours in the absence of light.

Serine incorporated C^{14} prior to glycine in tobacco leaves, as has been observed with Bryophyllum (13). Serine has been demonstrated to be precursor to glycine in animal tissues (6). Whether serine is synthesized in higher plants by a phosphorylated or non-phosphorylated pathway cannot be determined at the present time, since we have found both glycerate and phosphoglycerate labeled in the plant extracts.

Tolbert and Cohan (18) have shown that the major products formed from glycolate in barley and wheat leaves are glycine, serine and an unknown compound. There is a direct conversion of the glycolate to glycine and to the carboxyl and α-carbon of serine. The β-carbon of serine is formed from the α-carbon of glycolate. This evidence clearly establishes a direct biosynthetic pathway for the synthesis of glycine from glycolate. Therefore, it appears that there are two separate pathways present in leaves of higher plants for the biosynthesis of serine and glycine. It is difficult to assess the relative importance of the two pathways. Certainly in the dark, where CO_2 fixation in glycolate is not observed, radioactive serine arises from a 3-carbon precursor.

The striking qualitative similarity of the metabolic products involved with the dark fixation of CO_2 in excised leaves of *Nicotiana tabacum* and *Bryophyllum calycinum* are apparent from these studies. Although the rate of the initial CO_2 fixation in the absence of light is comparable and the mechanism of the initial carboxylation reaction appears to be identical, the succulents appear to have a unique mechanism for the storage of the synthesized organic acids which might account for the accumulation of a large quantity of the fixed carbon dioxide. We are not able to account for the difference at this time.

Summary

1. Excised leaves of *N. tabacum* have the ability to incorporate $C^{14}O_2$ in the dark into several organic and amino acids. These compounds have been identified by paper chromatography and radioautography.

2. The initial rate of dark CO_2 fixation in tobacco is more rapid than that of the succulent *B. calycinum*. However, the total amount of CO_2 fixed by tobacco after extended periods is much less.

3. The initial fixation of $C^{14}O_2$ appears to be mediated by the enzyme phosphoenolpyruvate carboxylase. The presence of this enzyme in tobacco leaves has been established.

4. The pattern of organic and amino acids which incorporate $C^{14}O_2$ suggest the operation of the Krebs cycle and concomitant transaminations. Serine seems to be the precursor to glycine in the dark.

5. No carbohydrates or phosphorylated sugars are labeled after extended periods of dark incorporation of $C^{14}O_2$. These findings suggest that light is needed to furnish the reducing power to reverse glycolytic processes.

We wish to acknowledge the valuable suggestions and assistance of Dr. John L. Webb in the preparation of this manuscript.

Literature Cited

1. Bandurski, R. S. and Greiner, C. M. Further studies on the enzymatic synthesis of oxaloacetate from phosphoenolpyruvate and carbon dioxide. Jour. Biol. Chem. 217: 137–150. 1953.
2. Benson, A. A. and Calvin, M. The path of carbon in photosynthesis VIII. Respiration and photosynthesis. Jour. Exptl. Bot. 1: 63–68. 1950.
3. Bradbeer, J. W., Ranson, S. L. and Stiller, M. Malate synthesis in crassulacean leaves. The distribution of C^{14} in malate of leaves exposed to $C^{14}O_2$ in the dark. Plant Physiol. 33: 66–69. 1958.
4. Claggett, C. O., Tolbert, N. E. and Burris, R. H. Oxidation of α-hydroxyacids by enzymes from plants. Jour. Biol. Chem. 178: 977–987. 1949.
5. Gibbs, M. The position of C^{14} in sunflower leaf metabolites after exposure of leaves to short period photosynthesis and darkness in an atmosphere of $C^{14}O_2$. Plant Physiol. 26: 549–556. 1951.
6. Ichihara, A. and Greenberg, D. M. Further studies on the pathway of serine formation from carbohydrate. Jour. Biol. Chem. 224: 331–340. 1957.
7. Isherwood, F. A. and Cruickshank, D. H. Chromatographic separation and analysis of mixtures of pyruvic, oxaloacetic and α-ketoglutaric acids. Nature 173: 121–122. 1954.
8. Kenton, R. H. and Mann, P. J. G. Hydrogen peroxide formation in oxidations catalysed by plant α-hydroxyacid oxidase. Biochem. Jour. 52: 130–134. 1952.
9. Levy, A. L. and Chung, D. Two dimensional chromatography of amino acids on buffered paper. Anal. Chem. 25: 396–399. 1953.
10. Mazelis, M. and Vennesland, B. Carbon dioxide fixation into oxaloacetate in higher plants. Plant Physiol. 32: 591–599. 1957.
11. Ranson, S. L. The use of isotopically labelled carbon dioxide in experiments bearing on two matters in the main body of this paper. Physiological studies on acid metabolism in green plants III. Further evidence of CO_2 fixation during dark acidification of plants showing crassulacean acid metabolism. New Phytologist 53: 28–30. 1954.
12. Saltman, P., Kunitake, G., Spolter, H. and Stitt, C. The dark fixation of CO_2 by succulent leaves: The first products. Plant Physiol. 31: 464–468. 1956.
13. Saltman, P., Lynch, V. H., Kunitake, G. M., Stitt, C. and Spolter, H. The dark fixation of CO_2 by succulent leaves: Metabolic changes subsequent to initial fixation. Plant Physiol. 32: 197–200. 1957.
14. Sen, S. P. and Leopold, A. C. Influence of light and darkness upon carbon dioxide fixation. Plant Physiol. 31: 323–329. 1956.
15. Smillie, R. M. Enzymic activities of sub-cellular particles from leaves. I. The occurrence of mitochondria in green leaves of the pea plant. Australian Jour. Biol. Sci. 9: 81–91. 1956.
16. Stiller, M. D., Neal, G. E. and Beevers, H. CO_2 fixation during the conversion of fat to carbohydrate in the castor bean. Plant Physiol. 33 Suppl.: xxxiv. 1958.
17. Stutz, R. E. and Burris, R. H. Photosynthesis and metabolism of organic acids in higher plants. Plant Physiol. 26: 226–243. 1951.
18. Tolbert, N. E. and Cohan, M. S. Products formed from glycolic acid in plants. Jour. Biol. Chem. 204: 649–654. 1953.
19. Vickery, H. B. and Abrahams, M. D. The metabolism of the organic acids of tobacco leaves. III. Effect of culture of excised leaves in solution of oxalate. Jour. Biol. Chem. 186: 411–416. 1950.
20. Vickery, H. B. and Palmer, J. K. The metabolism of the organic acids of tobacco leaves. X. Effect of culture of excised leaves in solution of fumarate and maleate. Jour. Biol. Chem. 218: 225–239. 1956.
21. Vickery, H. B. and Pucher, J. K. The metabolism of organic acids of tobacco leaves. XIII. Effect of culture of excised leaves in solutions of potassium bicarbonate. Jour. Biol. Chem. 227: 69–82. 1957.